NOVEL PEPTIDE ANALOGS FOR THE TREATMENT OF CANCER

FIELD OF THE INVENTION

This invention relates to novel peptide analogs of vasoactive intestinal peptide, somatostatin, bomebesin and Substance P. This invention also relates to the use of the novel peptide analogs for the treatment of cancer.

BACKGROUND OF THE INVENTION

Neuropeptide analogs are increasingly used in the treatment of cancer. The neuropeptides vasoactive intestinal peptide and bombesin exert physiological effects by binding to specific receptors present on cells in the gastrointestinal tract and central nervous system.

Bombesin is an amphibian peptide that has a structure closely related to that of several mammalian peptides, including gastrin releasing peptide (GRP) and Neuromedins B and C. Bombesin was discovered in 1970 and is a potent smooth muscle contracting agent of nonmammalian origin first isolated from amphibian skin (Erspamer et al. J. Pharm. Pharmacol. 22:275 (1970)). Bombesin, GRP and related peptides exert their *in vivo* effects by binding to specific receptors present on cells of the gastrointestinal tract, the central nervous system and on tumors.

VIP is a 28-amino acid neuropeptide, which has been implicated as a major growth promoting factor during embryonic growth. In cancer cells, previous studies have implied that VIP can serve as an autocrine growth factor in lung tumors (Gozes et al. Biomed. Res. 13 (Suppl. 2) 37, (1992)). By blocking the binding of VIP to its receptor these analogs inhibit the growth of tumor cells that respond to the growth-promoting action of VIP.

In U.S. Patent Application 08/727,679, we have described the role of neuropeptides in cancer. High affinity and moderate affinity receptors for vasoactive intestinal peptide and somastostatin, high affinity receptors for bombesin and moderate affinity receptors for substance P were demonstrated on human colon adenocarcinoma cells. It was further demonstrated that peptide analogs to the above neuropeptides could actively and selectively induce cell death in the cancer cells. A formulation of peptide combination termed MuJ-7 has also been described which

causes tumor regression in xenotransplanted nude mice. The individual constituent peptides of Mul-7 were demonstrated to have anticancer activity.

SUMMARY OF THE INVENTION

This invention provides novel peptides. These peptides are useful for the treatment of cancer. The invention relates to novel bombesin analogs which act as antagonists of bombesin or related peptides such as gastrin releasing peptide. By blocking the binding of bombesin-like peptides to their receptors, these antagonists block the physiological effects of these peptides and inhibit the growth of tumor cells that respond to growth-promoting action of bombesin. Thus these antagonists have therapeutic use in the treatment or prevention of cancer and in controlling physiological effects in gastrointestinal disorders and in modulating responses of the central nervous system.

The invention also relates to novel vasoactive intestinal peptide analogs which act as antagonists of VIP by blocking the binding of VIP to its cognate receptors.

The invention further relates to the designing and testing of novel structural analogs of Substance P and somatostatin, and analogs of VIP receptor binding inhibitor and bombesin antagonist which have been designed to render conformational constraints and higher stability to the peptides while maintaining their anticancer activity. Substitution and/or deletions have been incorporated into for example, VIP_2 and BOM_1 sequences bearing in mind not to alter amino acids known to offer conformational constraint and stability to the peptide. In order to introduce conformational constraints, unusual amino acids such as cyclic and acyclic dialkylated glycines have been incorporated into the peptide backbone. Preferably the cyclic ring is a C_3-C_8 ring and the number of carbon atoms in the alkyl group is from 1 to 6 (methyl to hexyl). Examples of such amino acids are Aib, MeLeu, Di-ethylglycine and its higher homologs, and 1-amino cycloalkane carboxylic acids. Aib represents α-amino-isobutyric acid.

DETAILED DESCRIPTION OF THE INVENTION

The VIP receptor binding inhibitor VIP_2 (Leu-Met-Tyr-Pro-Thr-Tyr-Leu-Lys) (SEQ ID NO:1) has been shown in our previous studies to be a selective cytotoxic peptide for cancer cells having receptors for vasoactive intestinal peptide.

Novel peptides that have conformational constraints and resist enzymatic degradation are formed by replacing any of the amino acids of the sequence Leu-Met-Tyr-Pro-Thr-Tyr-Leu-Lys with Dxg. Dxg represents cyclic and acyclic dialkylated glycines where the cyclic ring is a C_3-C_8 ring and the number of carbon atoms in the alkyl group is from 1 to 6 (methyl to hexyl). Examples are Aib, MeLeu, Di-ethylglycine and its higher homologs, and 1-amino cycloalkane carboxylic acids. Aib represents α-amino-isobutyric acid.

Novel peptides include:

DT-11 Aib-Met-Tyr-Pro-Thr-Tyr-Aib-Lys-OH (SEQ ID NO:2)

DT-12 D-Leu-Met-Tyr-Pro-Thr-Tyr-Aib-Lys-OH (SEQ ID NO:3)

DT-13 Leu-Met-Tyr-Pro-Thr-D-Tyr-Leu-Lys-OH (SEQ ID NO:4)

DT-14 Leu-Met-Tyr-Pro-Thr-Tyr-D-Leu-Lys-OH (SEQ ID NO:5)

DT-15 Leu-Met-D-Tyr-Pro-Thr-Tyr-D-Leu-Lys-OH (SEQ ID NO:6)

DT-16 D-Leu-Met-Tyr-Pro-Thr-Tyr-D-Leu-Lys-OH (SEQ ID NO:7)

DT-18 Aib-Met-Tyr-Pro-Thr-Tyr-Dxg-Lys-OH (SEQ ID NO:8)

DT-19 D-Leu-Met-Tyr-Pro-Thr-Tyr-Dxg-Lys-OH (SEQ ID NO:9)

where Dxg and Aib are as defined above.

The bombesin antagonist BOM_1 (D-Phe-Gln-Trp-Ala-Val-Gly-His-Leu-NHEt) (SEQ ID NO:10) has been shown in our previous studies to be a selective cytotoxic peptide for cancer cells having receptors for bombesin. Novel peptides that have conformational constraints and resist enzymatic degradation are formed by replacing any of the amino acids of the sequence D-Phe-Gln-Trp-Ala-Val-Gly-His-Leu-NHEt (SEQ ID NO:10) with Dxg where Dxg is as defined above. Leucine may be replaced with isoleucine and tryptophan may be replaced by D-tryptophan.

Novel peptides include:

DT-21 D-Phe-Gln-Trp-Ala-Val-Gly-His-Leu-NH_2 (SEQ ID NO:11)

DT-22 D-Phe-Gln-Trp-Ala-Val-Aib-His-Leu-NH_2 (SEQ ID

NO:12)

DT-23 D-Phe-Gln-Trp-Aib-Val-Gly-His-Leu-NH_2 (SEQ ID NO:13)

DT-24 D-Phe-Gln-Trp-Ala-Val-Aib-His-Leu-NH_2 (SEQ ID NO:14)

DT-25 D-Phe-Gln-Trp-Ala-Val-Gly-His-Ile-NH_2 (SEQ ID NO:15)

DT-26 D-Phe-Gln-Trp-Aib-Val-Gly-His-Ile-NH_2 (SEQ ID NO:16)

DT-27 D-Phe-Gln-Trp-Ala-Val-Aib-His-Ile-NH_2 (SEQ ID NO:17)

wherein Aib represents alpha-amino isobutyric acid.

The Substance P analog (D-Arg-Pro-Lys-Pro-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-Leu-NH_2 (SEQ ID NO:18)) has been shown in our previous studies to be a selective cytotoxic peptide for cancer cells having receptors for Substance P. Novel peptides that have conformational constraints and resist enzymatic degradation are formed by replacing any of the amino acids of the sequence D-Arg-Pro-Lys-Pro-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-Leu-NH_2 (SEQ ID NO:18)) with Dxg or Aib. Dxg and Aib are as defined above. Analogs may be 5 to 11 amino acids.

Novel peptides include:

DT-31 Aib-Met-Gln-Trp-Phe-Aib-NH_2 (SEQ ID NO:19)

DT-32 Dxg-Met-Gln-Trp-Phe-Aib-NH_2 (SEQ ID NO:20)

DT-33 D-Leu-Met-Gln-Trp-Phe-Aib-NH_2 (SEQ ID NO:21)

DT-34 D-Arg-Pro-Lys-Pro-Aib-Gln-D-Trp-Phe-D-Trp-Aib-Leu-NH_2 (SEQ ID NO:22)

DT-35 Arg-Pro-Aib-Pro-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-Leu-NH_2 (SEQ ID NO:23)

where Dxg and Aib are as defined above.

The somatostatin analog (Ala-Gly-Cys-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser-D-Cys (disulfide bridges: 3-14) (SEQ ID NO:24) has been shown in our previous studies to be a selective cytotoxic peptide for cancer cells having receptors for Somatostatin. Novel peptides that have conformational constraints and

resist enzymatic degradation are formed by replacing any of the amino acids of the sequence (Ala-Gly-Cys-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser-D-Cys (disulfide bridges:3-14) (SEQ ID NO:24) with Dxg or Aib where Dxg and Aib are as defined above.

A novel peptide is:

DT-61 Ala-Aib-Cys-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-D-Ser-Cys (3-14 disulfide bond) (SEQ ID NO:25)

where Aib represents α-amino isobutyric acid.

The somatostatin analog (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2) (SEQ ID NO:26) has been shown in our previous studies to be a selective cytotoxic peptide for cancer cells having receptors for Somatostatin. Novel peptides that have conformational constraints and resist enzymatic degradation are formed by replacing any of the amino acids of the sequence D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (SEQ ID NO:26) with Dxg or Aib where Dxg and Aib are as defined above.

A novel peptide is:

DT 71 D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Aib-Thr-NH_2 (SEQ ID NO:27)

where Aib represents alpha-amino isobutyric acid.

The present invention is further described in detail with reference to the following examples, which are given for purpose of merely illustrating the invention without limiting it.

EXAMPLE 1

The cytotoxic activity of the peptides synthesized was tested on eight human tumor cell lines namely HT-29, SW620, PTC (all colon), PA-1 (ovary), A549 (lung), HBL100 (breast), MOLT-4 (leukemia) and DU145 (prostate). The tumor cells were collected at exponential growth phase and resuspended in medium (1.5×10^6 cells/ml in RPMI 1640 containing 10% FBS). 150 µl of medium was added to the wells of a 96-well tissue culture plate (Nunc, Denmark) followed by 30 µl of cell suspension. The plate was left in an incubator (37°C, 5% CO_2) overnight. 20 µl of the pepide (10^{-7} to 10^{-10}M concentration) was added to marked wells of the 96 well plate. Each concentration was plated in triplicate. 20 µl of medium alone was added to control wells while wells without cells served as blanks. A total volume of 200 µl

was ensured in each well and the plate was left in the incubator (37°C, 5% CO_2). After 72 hours of incubation an MTT assay was performed and percentage cytotoxicity was calculated with respect to control cells. DT-1: bombesin antagonist, DT-2: VIP receptor binding inhibitor, DT-3: substance P analog, DT-4 and DT-7: Somastatin analogs.

TABLE 1

PERCENT CYTOTOXICITY OF DT-1 ANALOGS ON VARIOUS TUMOR CELL LINES

Cell line	DT11	DT12	DT13	DT14	DT15	DT16	DT18	DT19
PA-1	32	26	23	14	25	22	31	34
PCT	16	24	20	0	23	18	14	10
MOLT-4	30	42	39	30	33	31	36	23
HBL100	24	19	27	19	18	2	24	27
A549	29	36	32	42	37	33	27	25
SW620	13	0	18	12	28	22	26	33
HT29	0	7	6	6	11	20	38	30
DU145	7	19	1	5	10	0	18	24

TABLE II

PERCENT CYTOTOXICITY OF DT-2 ANALOGS
ON VARIOUS TUMOR CELL LINES

Cell line	DT21	DT22	DT23	DT24	DT25	DT26	DT27
PA-1	23	24	16	33	21	24	26
PTC	18	12	15	23	9	25	12
MOLT-4	25	17	29	8	23	20	13
HBL100	27	14	33	32	25	6	16
A549	16	22	23	30	25	13	18
SW620	25	33	34	38	31	38	32
HT29	24	35	43	44	40	27	28
DU145	10	22	25	32	33	24	0

TABLE III

PERCENT CYTOTOXICITY OF DT-3 ANALOGS
ON VARIOUS TUMOR CELL LINES

Cell line	DT31	DT32	DT33	DT34	DT35
PA-1	24	ND	ND	ND	ND
PTC	21	32	23	16	18
MOLT-4	30	32	37	26	20
HBL100	15	16	15	21	15
A549	23	19	21	23	20
SW620	14	ND	ND	ND	ND
HT29	30	13	18	9	26
DU145	25	ND	ND	ND	ND

TABLE IV

PERCENT CYTOTOXICITY OF DT-6 ANALOGS
ON VARIOUS TUMOR CELL LINES

Cell line	DT61
PA-1	45
PTC	22
MOLT-4	34
HBL100	26
A549	28
SW620	26
HT29	35
DU145	29

TABLE V

PERCENT CYTOTOXICITY OF DT-7 ANALOGS
ON VARIOUS TUMOR CELL LINES

Cell line	DT71
PA-1	ND
PTC	19
MOLT-4	37
HBL100	23
A549	18
SW620	ND
HT29	19
DU145	ND

EXAMPLE 2

A 0.5 mL of 2000 ppm of VIP_2 was mixed with 1.0 ml of freshly prepared liver homogenate to obtain a concentration of 1000 ppm. Sample preparations were incubated at 37°C and after time intervals of 0, 2, 5, 10, 20 and 30 minutes, 200 µl of the preparation was aliquoted and precipitated with equal volumes

of acetonitrile. In case of BOM_1 analogs, the sample preparations with a final concentration of 1000 ppm were incubated at 37°C and after time intervals of 0, 15, 30, 60, 90, 120 and 150 minutes, 200 µl of the preparation was aliquoted and precipitated with equal volumes of acetonitrile. The precipitate was pelleted by centrifugation at 10,000 g for 5 minutes and supernatant was analyzed by HPLC. The percentage increase in the half-life of DT1 (VIP receptor binding inhibitor) and DT2 (bombesin antagonist) analogs with reference to DT1 and DT2 respectively, as estimated by the mouse liver homogenate study is shown in Tables VI and VII respectively.

TABLE VI

HALF LIFE OF DT-1 ANALOGS WITH REFERENCE TO DT-1
AS DETERMINED BY THE MOUSE LIVER HOMOGENATE STUDY

Peptide	Half-life (minutes)
DT-1	4.9
DT-11	15.4
DT-12	18.1

TABLE VII

HALF LIFE OF DT-2 ANALOGS WITH REFERENCE TO DT-2
AS DETERMINED BY THE MOUSE LIVER HOMOGENATE STUDY

Peptide	Half-life (minutes)
DT-2	12.6
DT-22	15.06
DT-23	57.7
DT-24	38.5
DT-26	114.5
DT-27	292.5

PREPARATION OF MOUSE LIVER HOMOGENATE

1. Healthy Balb/c mouse was sacrificed and dissected to expose liver.
2. The pulmonary artery was severed to drain blood and cold saline

was perfused through the portal vein until the liver becomes pale white.

3. The liver was excised, minced and passed through 60# sieve.

4. 1.15% w/v KCl -0.01M phosphate buffer,(pH 7.4) was added to make 20% w/v homogenate tht was centrifuged at 4500 g for 15 min.

5. The supernatant was recovered and further centrifuged at 10,000 g to clarify the homogenate.